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Synthesis and biological evaluation of 1,2,4-trisubstituted imidazoles and 1,3,5-trisubstituted pyrazoles as inhibitors of transforming growth factor β type 1 receptor (ALK5)

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ABSTRACT

Two series of nitrogenous heterocycle compounds—1,2,4-trisubstituted imidazoles and 1,3,5-trisubstituted pyrazoles have been synthesized and evaluated for their ALK5 inhibitory activity and cytotoxicity in TGFβ-Smad2 assay and MTT assay, respectively. The ALK4/5/7 inhibitory activity of some compound was also evaluated in ALK4/5/7 autophosphorylation assays. Compounds **6c** and **14c** showed relatively potent ALK5 inhibition while weak cytotoxicity. At the same time, compounds **6c** and **14c** display relatively better ALK5 selectivity versus ALK4/ALK7 (nearly 10-fold) compared with SB431542, a well known ALK5 inhibitor. Compound **6g2** proved to be a moderately selective ALK4 inhibitor versus ALK5 and ALK7 (>10-fold). The binding mode of **14c** generated by flexible docking study shows that **14c** fits well into the site cavity of ALK5 by forming several tight interactions.

Progressive fibrosis in the major organs such as kidney, liver, lung, heart, and skin is both a major cause of suffering and death and an important contributor to the cost of health care. Transforming growth factor β (TGFβ) has been postulated to play a central role in pathological fibrosis.[1–5] The complex function of TGFβ is dependent on the activation of two highly conserved single trans-membrane serine/threonine kinases, the type I receptor [TβRI or activin-like kinase 5 (ALK5)] and type II receptor (TβRII), respectively. Upon TGFβ binding, TβRII phosphorylates threonine residues in the GS region of the ligand-occupied TβRI. The activated TβRI in turn phosphorylates Smad2/Smad3 proteins at the C-terminal SSXS-motif, thereby causing Smad2/Smad3 dissociation from the receptor and heteromeric complex formation with Smad4. Then the Smad complexes translocate to the nucleus and regulate gene respouns.[6] Therefore, it becomes evident that inhibition of phosphorylation of Smad2/Smad3 by ALK5 could reduce TGFβ-induced pathological fibrosis.

The 2-pyridyl substituted nitrogenous heterocycle small molecules (e.g., SB-431542, SB-505124, as shown in Fig. 1) are a novel class of selective inhibitors of ALK5. SB-431542 and SB-505124 selectively inhibit the in vitro phosphorylation of immobilized Smad3 with an IC_{50} of 94 nM, and 47 nM, respectively.[7,8] To develop novel type of ALK5 inhibitor, we have designed and synthesized a series of 1,2,4-trisubstituted imidazoles and 1,3,5-trisubstituted pyrazoles to mimic the structure of SB-431542 and SB-505124. In additional to the central core modifications, new type of substitute group were also investigated. It is particular interest to us whether novel type of substitute groups other than the 2-pyridyl group, which is necessary to traditional ALK5 inhibitor, may work as well or better. So we attempted to replace the pyridine moiety with 2-fluorophenyl, 2-methoxyphenyl, 2-hydroxylphenyl, thiazole-2-yl or 2-trifluoromethylphenyl. Likewise, replacement of the benzo[1,3]dioxol-5-yl and 4-fluorophenyl with other group, such as 3,4-dimethoxyphenyl, 3,4-dihydroxyphenyl, benzo[1,4]-dioxol-5-yl and 3-fluoro-4-methoxyphenyl, were also practiced to enrich the versatility of the substituent. The present Letter describes our efforts in this field, and a limited exploration of the SAR of the synthesized compounds was also discussed.

The 1,3,5-tri-substituted pyrazoles were prepared as shown in Scheme 1. Treatment of the solution of substituted benzaldehyde (**1**) and 4-acetylbenzonitril (**2**) in EtOH in the presence of NaOH catalysts gave substituted chalcones (**3**) in good yield.[9] Reaction

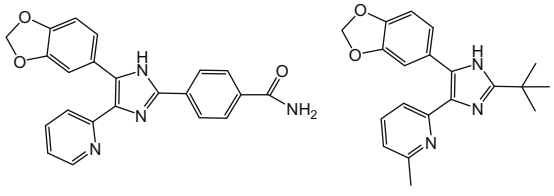

Figure 1. The structure of SB431542 and SB505124.

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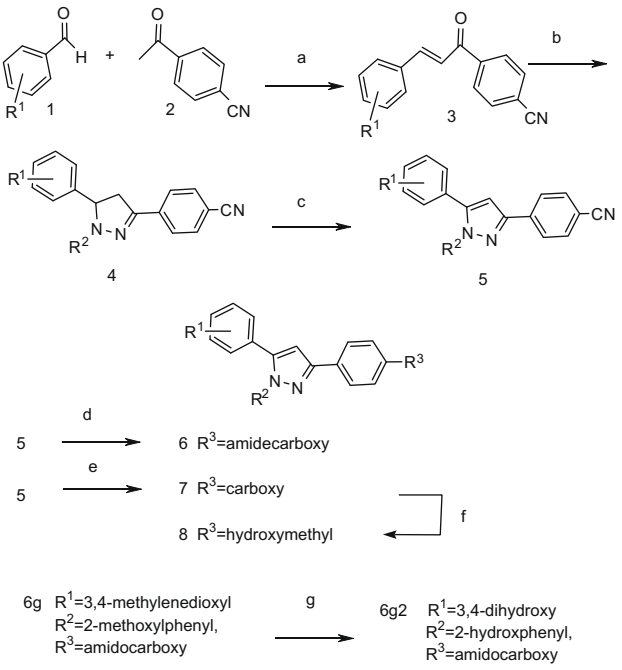
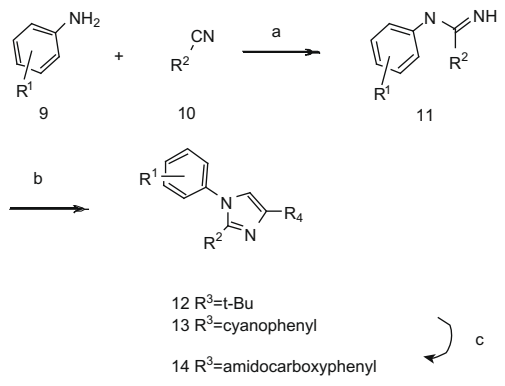


Scheme 1. Reagents and conditions: (a) NaOH,EtOH; (b) 2-fluorophenylhydazin, EtOH, reflux; (c) MnO_2/SiO_2, CH_2Cl_2; (d) KOH, *t*-BuOH, reflux; (e) NaOH, EtOH/H_2O, reflux; (f) $LiAlH_4$, THF; (g) BBr_3/CH_2Cl_2.

of substituted chalcones (**3**) and arylhydrazine or hydrochloric arylhydrazine in ethanol produced the 1,3,5-trisubstituted pyrazolins (**4**)[10], which can be oxidated to corresponding 1,3,5-tri-substituted-pyrazoles (**5**) by MnO_2/SiO_2.[11] Conversion of the nitrile functionality of 5 to carboxamide was accomplished by treatment with KOH powder in *t*-BuOH under reflux to afford compounds **6**.[12] While under the condition of NaOH/ethanol/water, the nitrile group can be converted to carboxyl giving compound **7**. The carboxyl was reduced to hydroxymethyl by treatement with $LiAlH_4$ to give compounds **8**. Treatment of **6g** with BBr_3 in CH_2Cl_2 offered the trihydroxyl substituted product **6g2**[13]

The 1,2,4-trisubstituted imidazoles were prepared as shown in Scheme 2. Coupling of substituted cyanobenzene (**9**) with substituted aniline (**10**) in a mixture of THF in the presence of $NaN(SiMe_3)_2$ gave the intermediates substituted amidines(**11**).[14] The amidines **11** condensed with 4-bromoacetylbenzonitrile or 1-bromo-3,3-dimethyl-butan-2-one offered the corresponding 1,2,4-trisubstituted imidazoles (**12** or **13**). The nitrile group of **13** can be converted to



Scheme 2. Reagents and conditions: (a) $NaN(SiMe_3)_2$,THF; (b) 4-bromoacetylbenzonitrile or 1-bromo-3,3-dimethyl-butan-2-one, $NaHCO_3$, i-PrOH; (c) KOH, *t*-BuOH, reflux.

carboxamide by treatment with KOH powder in *t*-BuOH under reflux to afford compounds **14**.

To investigate whether these potential inhibitors could inhibit TGFβ-induced downstream transcriptional activation to ALK5 signaling, cell based TGFβ-Smad2 assay (Bioimage) was used for this analysis (Tables 1 and 2).[15] In order to explore the kinase inhibition activities against ALK5 and its two close relative ALK4 and ALK7, their ALK4/5/7 inhibition activities were also evaluated by kinase based autophosphorylation assay[16] (Table 3). In addition to this, the cytotoxicities of some active compounds were also determined in MTT assay in HLF cell (Tables 1 and 2).[17] A standard compound, SB431542, was used for the calibration or comparison of result in all above assays.

Exploration of the SAR of our compounds revealed that the enzyme potency of our compounds did not match well correlate with the observed cellular activities. Good alk5 inhibition activity was observed with the 1,3,5-trisubstituted pyrazoles in kinase based autophosphorylation assay (Table 3), while better overall cellular activity was observed with the 1,2,4-trisubstituted imidazoles in cell based TGFβ-Smad2 assay (Table 2). These finding suggest that the 1,2,4-trisubstituted imidazoles may have better cellular permeability than the 1,3,5-trisubstituted pyrazoles.

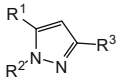
Replacement of the pyridine-2-yl group with 6-methyl-pyridine-2-yl and 2-fluoro-phenyl led to improvement of activity. Compound **14c** displayed significant ALK5 inhibition (69% inhibition at 1 μM, Table 2), which is compared to that of SB431542 (76% inhibition at 1 μM), in cell base assay. Compound **6c** showed even better ALK5 inhibitory activity (53.9% at 0.1 μM and 91.2% at 1 μM, Table 3) than that of SB431542 (14.6% at 0.1 μM and 75.1% at 1 μM) in kinase based autophosphorylation assay. Introduction of thiazole-2-yl, 2-trifluoromethyl-phenyl or 2-methoxyphenyl to replace the pyridine-2-yl group causes a loss of activity of kinase inhibition. Replacement benzo[1,3]dioxol-5-yl with 4-fluorophenyl, benzo[1,4]dioxol-6-yl or 3-fluoro-4-methoxyphenyl resulted in a decline of activity, while the 3,4-dimethoxyphenyl retained the activity. Interestingly, analogue **6g2** with 3,4-dihydroxyphenyl and 2-hydroxyxyphenyl substitute displayed relative good ALK5 inhibition. The SAR of R3 observed in our work were the same as reported.[18] 4-amidocarboxphenyl containing analogues displayed the most potent activity compared with analogues containing 4-cyanophenyl, 4-carboxyphenyl or 4-hydroxymethylphenyl and *t*-butyl.

It is worth of mentioning that the ALK5 inhibition of SB431542 is very close to the ALK4 inhibition and ALK7 inhibition according to kinase based assay as shown in Table 3. This observation is in line with the report by Gareth[8]. Compounds **6c** and **14c** showed relatively better ALK5 selectivity versus ALK4/ALK7 (nearly 10-fold) compared with SB431542. Some compounds showed relatively good ALK4 or ALK7 inhibitory activity against ALK5. For example, compounds **6a**, **5c**, **6h**, **6g2**, **13c** and **14a** maintained even better ALK4 inhibitory activities than that of SB431542. Herein, compound **6g2** proved to be a moderately selective ALK4 inhibitor versus ALK5 and ALK7 (>10-fold). On the other hand, compounds **5c**, **6c**, **6h** and **14d** possessed good ALK7 autophosphorylation inhibitory activity. It should be noted that the kinase domains of ALK4 and ALK5 are 89.3% identical, and those of ALK5 and ALK7 are 82.4% identical.[8] Therefore, it is very difficult for small molecule inhibitors to distinguish between these closely related family members.

Besides, nearly all our compounds exhibited weaker cytotoxicity against human lung fibroblast (HLF) compared with SB431542 at concentration of 30 μM.

To account for the SAR of our compounds, we built a docking model of ALK5/**14c** complex based on the X-ray structure of ALK5 complexed with LY580276 (PDB entry 1RW8).[19,20] As demonstrated in Figure 2, Compound **14c** is well superimposed over the

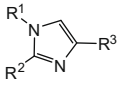
Table 1
The synthesized 1,3,5-trisubstituted pyrazoles and their ALK5 inhibition activity as well as cytotoxicity



Compounds	R^1	R^2	R^3	ALK5 inhibition (%, 1 μM)[a]	Cytotoxicity (30 μM)[d]
5a	Benzo[1,3]dioxol-5-yl	Pyridin-2-yl	4-Cyanophenyl	NA[b]	ND[c]
6a			4-Amidocarboxphenyl	15.4	26.7
5b		2-Fluoro-phenyl	4-Cyanophenyl	9.0	ND
6b			4-Amidocarboxphenyl	26.0	ND
7b			4-Carboxyphenyl	9.6	5.4
8b			4-Hydroxymethylphenyl	NA	ND
5c		6-Methyl-pyridin-2-yl	4-Cyanophenyl	32.0	15.4
6c			4-Amidocarboxphenyl	45.3	5.6
6d		Thiazole-2-yl	4-Amidocarboxphenyl	NA	ND
6e		2-Trifluoromethyl phenyl	4-Amidocarboxphenyl	NA	ND
6f		Phenyl	4-Amidocarboxphenyl	NA	ND
6g		2-Methoxyphenyl	4-Amidocarboxphenyl	NA	ND
5i	3, 4-Dimethoxy phenyl	Pyridin-2-yl	4-Cyanophenyl	NA	ND
6i			4-Amidocarboxphenyl	14.7	5.3
5h		2-Fluoro-phenyl	4-Cyanophenyl	8.3	ND
6h			4-Amidocarboxphenyl	33.9	19.4
6j	Pyridin-2-yl	Benzo[1,3]dioxol-5-y	4-Amidocarboxphenyl	NA	ND
5k	2-Fluoro-phenyl		4-Cyanophenyl	6.0	57.5
6k			4-Amidocarboxphenyl	19.0	0.52
6l	4-Fluorophenyl	Pyridin-2-yl	4-Amidocarboxphenyl	NA	23.7
6m		2-Methoxyphenyl	4-Amidocarboxphenyl	NA	ND
6m2		2-Hydroxyphenyl	4-Amidocarboxphenyl	NA	ND
6n		2-Fluoro-phenyl	4-Amidocarboxphenyl	24.4	19.54
6g2	3,4-Dihydroxy-phenyl	2-Hydroxyphenyl	4-Amidocarboxphenyl	21.0	48.9
SB431542				24.2(0.1 μM), 82.5(1 μM)	55.0

[a] Activity is given as the mean of triplicated determinations relative to control incubations with DMSO vehicle.
[b] NA = no significant inhibition.
[c] ND = not detected.
[d] Determined by using the MTT method.

Table 2
The synthesized 1,2,4-trisubstituted-imidazoles and their ALK5 inhibition activity as well as cytotoxicity



Compounds	R^1	R^2	R^3	ALK5 inhibition[a] (%, 1 μM)	Cytotoxicity [d] (30 μM)
14a	Benzo[1,3]dioxol-5-yl	Pyridin-2-yl	4-Amidocarboxphenyl	20.9	21.6
14b	Benzo[1,4]dioxol-5-yl	6-Methyl-pyridin-2-yl	4-Amidocarboxphenyl	8.1	21.0
13c	Benzo[1,3]dioxol-5-yl		4-Cyanophenyl	41.4	4.0
14c			4-Amidocarboxphenyl	69.0	14.9
12c			*t*-Bu	24.7	7.3
13d	4-Fluorophenyl		4-Cyanophenyl	NA[b]	20.2
14d			4-Amidocarboxphenyl	37.8	19.1
12d			*t*-Bu	NA	59.0
13e	Benzo[1,4]dioxol-6-yl		4-Cyanophenyl	21.1	28.0
14e			4-Amidocarboxphenyl	28.2	0.7
12e			*t*-Bu	NA	ND[c]
13f	3-Fluoro-4-methoxyphenyl		4-Cyanophenyl	10.5	57.0
14f			4-Amidocarboxphenyl	10.8	9.1
12f			*t*-Bu	NA	22.1
14g	Benzo[1,3]dioxol-5-yl	2-Fluoro-phenyl	4-Cyanophenyl	15.8	ND
13g			4-Amidocarboxphenyl	42.4	12.3
SB431542				24.2(0.1 μM), 82.5(1 μM)	55.0

[a] Activity is given as the mean of triplicated determinations relative to control incubations with DMSO vehicle.
[b] NA = no significant inhibition.
[c] ND = not detected.
[d] Determined by using the MTT method.

X-ray pose of LY580276, occupying the binding site of ATP and the nearby 'selectivity pocket'. The benzo[1,3]dioxol-5-yl moiety of **14c** interacts with the backbone of amino acids (residues 281–283) that links the N and C terminal domains of the kinase (hinge region), a region normally occupied by the adenine ring of ATP. One hydrogen bond exists between the O atom at the 1-position of the

Table 3
ALK4/5/7 Inhibition activities of selected compounds determined by phosphorylation assay

Compounds	Inhibition (%)					
	ALK4		ALK5		ALK7	
	0.1 μM	1 μM	0.1 μM	1 μM	0.1 μM	1 μM
SB431542	25.7	69.4	14.6	75.1	8.9	54.0
6a	70.0	74.7	6.9	17.2	35.5	57.3
6b	15.5	50.7	9.9	24.5	22.7	39.1
5c	53.6	77.7	24.6	45.2	31.4	73.4
6c	39.4	66.8	53.9	91.2	4.2	64.0
6h	32.1	70.4	6.2	42.8	5.3	65.8
6k	3.2	41.9	30.7	55.4	50.9	57.4
6g2	73.8	93.7	8.8	30.3	34.8	56.5
14a	65.6	73.7	5.3	11.7	24.5	54.8
13c	45.5	76.9	2.5	21.0	33.9	44.6
14c	15.6	29.8	32.7	67.2	4.5	41.3
13g	25.0	34.5	0.4	15.5	16.2	35.1
14e	28.5	49.3	3.5	12.7	13.4	25.0
14d	13.3	36.6	2.7	14.1	30.5	63.6

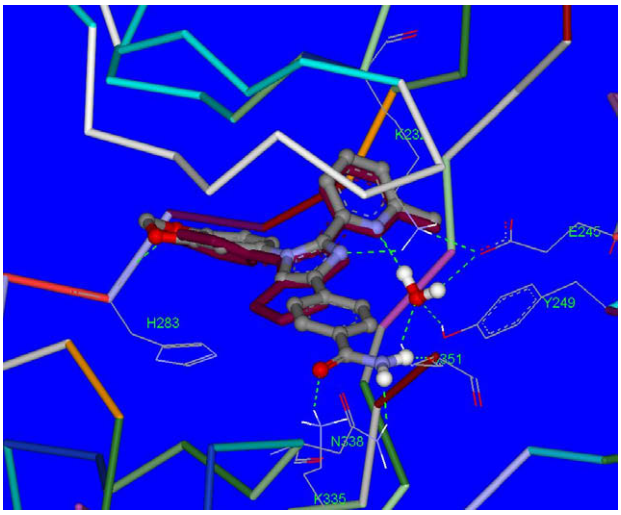


Figure 2. The binding mode of **14c** (rendered in capped stick and colored by atom type) observed in the docking model, in comparison with the X-ray pose of LY580276 (brown stick). Key amino acid residues within the ALK5-binding site are represented in line form. The trapped water in the X-ray structure is shown in red and white sphere. Green dotted lines are hydrogen bond interactions among 14c, the amino acid residues (His283, Lys232, Asp351, Asn338, Lys335, Glu245, Tyr249) and the trapped water.

benzo[1,3]dioxol-5-yl and the backbone of His283. An analogous hydrogen bond acceptor-donor pair occurs in many other kinase crystal structures and is also seen for N1 of adenine in ATP. In addition, residues Val219, His283, Ala230, and Leu340 make hydrophobic interaction with the benzene ring of benzo[1,3] dioxol-5-yl. The N3 (1) of the imidazole scaffold interacts with the side chain of Lys232 through a hydrogen bond. The 6-methyl pyridine ring is inserted into the so called 'selectivity pocket' formed between αC and β1–β4, making contacts with the following cluster of residues: Ala230, Lys232, Leu260, Leu278, Val279 and Ser280. The hydrogen bond to a water molecule hold in place by additional hydrogen bonds to Asp 351, Glu245 and Tyr249 is also maintained in this model, which is commonly observed in inhibitor containing pyridyl moiety in this position.[21,22] This pyridine N_1–H_2O can explain why a hydrogen bond acceptor group in this position is beneficial. The methyl group of the 6-methyl pyridyl moiety insert into a small hydrophobic cave formed by the side chain of Leu278, Tyr249 and Phe262. The 6-methyl-pyridin-2-yl analogues exhibited more potent ALK5 inhibition activity than the corresponding pyridin-2-yl analogues may contribute to this additional contact. The phenyl ring at the 4 position of imidazole makes hydrophobic interaction with Phe216, Val219 and Ala350. The amino group of the carboxamide interacts with Asp351 and Asn338 by hydrogen bonds. Another hydrogen bond is also formed between the carbonyl group of the carboxamide and Lys335. Conclusively, the binding model of **14c** generated by flexible docking studies shows that the structure of ligand fits well onto the binding cavity of ALK5 by forming tight interactions.

In this Letter, two series of nitrogenous heterocycle compounds—1,2,4-trisubstituted imidazoles and 1,3,5-trisubstituted pyrazoles have been synthesized and evaluated for their ALK5 inhibitory activity in TGFβ-Smad2 assay and cytotoxicity assay, respectively. The ALK4/5/7 inhibitory activity of some active compounds was also evaluated in ALK4/5/7 autophosphorylation assay. Some compounds showed moderate to high inhibition against ALK5, wherein compounds **6c** and **14c** showed relatively good ALK5 inhibition activities while weak cytotoxicity. At the same time, compounds **6c** and **14c** display relatively better ALK5 selectivity versus ALK4/ALK7 (nearly 10-fold) compared with SB431542. Compound **6g2** proved to be a moderately selective ALK4 inhibitor versus ALK5 and ALK7 (>10-fold). It is worth mentioning that 2-fluoro-phenyl was found to be a more favorite substituent compared with the pyridine-2-yl, which is necessary to the classic ALK5 inhibitors.

Acknowledgment

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.04.066.

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15. Cellular assays for measuring anti-TGF-β activity of ALK5 inhibitors. The activity of compounds weretested in EGFP-SMAD2 Assay (Bioimage, the assay is based on Redistribution™ technology to quantitate the intracellular translocation of an EGFP-SMAD2 fusion protein in a stably transfected CHO cell line. Following activation with TGF-β, EGFP-SMAD2 fusion protein hetero). To test anti-TGF-β activity of compounds, the cells were seeded in 96 well microplates at a concentration of 20,000 cells per well in 100 μL of serum-containing medium. The microplates were then placed for 24 h in a cell incubator at 37 °C, 5% CO_2 atm. The compounds dissolved in DMSO were then added at different concentrations (final concentration of DMSO 0.1%) for 30 min prior to the addition of recombiation TGF-β(3 ng/mL). After an hour incubation, the cells images wereacquired by Incell Analyzer 1000(GE Healthy). The data analysis using the Trafficking-Analysis Module (incell Analyzer 1000 Workstation version 1.4). Inhibition of compounds on TGF-β-SMAD2 were expressed as the inhibitory activity on ALK5.
16. Inhibition on ALK4/5/7 at molecular level. The kinase domain of ALK4/5/7 were cloned by PCR and expressed in baculovirus/Sf9 cells system. The protein 6-His tagged in the C terminus and purified by affinity chormatography using a Ni^{2+} column, and the obtained materials were used to assess compound activity in autophosphorylation assay. Purified ALK4/5/7 3 μg/mL was incubated with

different concentrations of compounds for 30 min at 37 °C, respectively. The reaction was then initiated by addition of 10–8 mol/L ATP and 10 μg/mL Smad3. After 3 h at 30 °C, phosphorylation was directly quantified by determining the consumed ATP using The ENLITEN® ATP Assay System(Promega).

17. HLF (Human Embryo Lung Fibrio lisis) Cell proliferation and cytotoxicity was determined by MTT assay. Approximately 3000 cells/well were seeded in 96-well plates (Coster) and incubated for 24 h before treatment with 0.1–5 mmol/L adenosine. The initial number of viable HLF cells at the time of treatment, termed $t = 0$, was then determined to correct for differences in starting cell number between experiments and to monitor changes in cell number over time. At the indicated times, MTT tetrazolium salt with phenazine methosulfate were added directly to the culture media and the cells were allowed to incubate for 2–3 h. Mitochondrial dehydrogenases of viable cells convert MTT into a color-dense formazan. All data presented in the present Letter were obtained from six independent experiments.
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20. The ligand and receptor preparation were performed with the SYBYL 6.9 software package (Tripos, Inc., St. Louis, MO). Ligand preparation: The structure of the ligand (**14c**) was prepared in MOL2 format using the sketcher module of SYBYL 6.9, Gasteiger–Huckel charges were assigned to the ligand atoms, and then energyminimized until converged to a maximum derivative of 0.001 kcal mol^{-1} $Å^{-1}$. The ligand was saved in the Tripos Mol2 format. Receptor preparation: The X-ray crystal coordinate of ALK5 complexed with LY580276 (PDB entry 1RW8) was retrieved from the Protein Data Bank (PDB), and all crystallographic water molecules were removed expect the one (HOH20, atom ID 2460) involved in H-bond with ligand inside the binding pocket. A correct atom assignment for Asn, Gln, and His residues was done, and hydrogen atoms were added. Partial atomic charges were computed using the Amber7FF02 force field. All heavy atoms were then fixed, and hydrogen atoms were minimized using the Amber7FF02 force field and a constant dielectric of 1, terminating at a gradient of 0.001 kcal mol^{-1} $Å^{-1}$. The receptor was saved in the Tripos Mol2 format. Docking: The docking experiments were performed with GOLD 3.1. Parameters were maintained as standard default GOLD 3.1 settings with the following exceptions. The active site was defined as all amino acid residues enclosed within 6.5 Å radius sphere centered by LY580276. The side chain of Lys232, Asp351 was set to be flexible. The water state of HOH20 (atom ID 2460) was set to 'on' and its orientaion was set 'spin'. Two hydrogen-bond constraint was added during calculations, guiding the O atom at the 1-position of the benzo[1,3]dioxol-5-yl and the N atom of the pyridine to interact with the backbone NH of His-283 and one proton of the water, respectively. Ten poses docking were saved, and the top-scoring pose was taken to be the favored pose.
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